

B/O Form PTO-1390		Transmittal Letter to the United States Designated/Elected Office (DO/EO/US) Concerning a Filing Under 35 USC 371	Attorney's Docket Number HOGL3001/REF
			U.S. Application Number (if known) 10/070412
International Application Number PCT/SE00/01767	International Filing Date September 13, 2000	Priority Date Claimed September 13, 1999	
Title of Invention DNA CONSTRUCT AND ITS USE			
Applicant(s) for DO/EO/US HOGLUND et al.			

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items under 35 USC 371:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 USC 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 USC 371.
3. ☒ This express request to begin national examination procedures (35 USC 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 USC 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed 35 USC 371(c)(2).
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 USC 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 USC 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 USC 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 USC 371(c)(4)). (☒ Executed ☐ Unexecuted).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 USC 371(c)(5)).

Items 11 to 16 below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
14. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and U.S.C. 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information: Sequence Listing and Application Data Sheet

JC13 Rec'd PCT/PTO 15 MAR 2002

Application Number (if Known) <div style="font-size: 1.5em; font-weight: bold;">10/070412</div>		International Application Number <div style="font-weight: bold;">PCT/SE00/01767</div>		Attorney's Docket Number <div style="font-weight: bold;">HOGL3001/REF</div>	
				Calculations	PTO USE ONLY
1. The following fees are submitted: Basic National Fee (37 CFR 1.492(a)(1)-(5)): <input checked="" type="checkbox"/> Neither International Preliminary Examination Fee (37 CFR 1.482) nor International Search Fee (37 CFR 1.445(a)(2)) paid to USPTO \$1040.00 <input type="checkbox"/> Search report has been prepared by the EPO or JPO \$890.00 <input type="checkbox"/> International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) \$710.00 <input type="checkbox"/> No International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) but International Search Fee paid to USPTO (37 CFR 1.445(a)(2)) \$740.00 <input type="checkbox"/> International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00					
ENTER APPROPRIATE BASIC FEE AMOUNT				\$ 1,040.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).					
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	20 -20 =	0	× \$18.00	\$ 0.00	
Independent Claims	1 -3 =	0	× \$84.00	\$ 0.00	
Multiple Dependent Claims (if applicable)			+ \$280.00		
TOTAL OF ABOVE CALCULATIONS					
Reduction by ½ for filing by small entity, if applicable. Small Entity Status is asserted pursuant to 37 CFR 1.27 for this application.				\$ 520.00	
SUBTOTAL				\$ 520.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).					
TOTAL NATIONAL FEE					
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property.				\$ 40.00	
TOTAL FEES ENCLOSED				\$ 560.00	
<div style="border: 1px solid black; padding: 5px;"> Amount to be: </div>				Refunded:	
				Charged:	

- a. ☒ A check in the amount of \$560.00 to cover the fees is enclosed.
- b. ☐ Please charge my **Deposit Account Number 02-0200** in the amount of \$_____ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to **Deposit Account Number 02-0200**. A duplicate copy of this sheet is enclosed.

Note: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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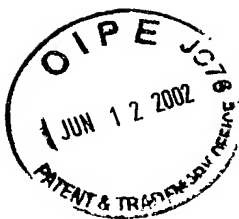
PATENT TRADEMARK OFFICE

Respectfully submitted,

DATE: March 15, 2002

Richard E. Fichter

Attorney for Applicant: Richard E. Fichter
 Registration Number: 26,382



STATEMENT

I, Brita Nilsson, hereby certify that the information recorded in computer readable form is identical to the written sequence listing as filed with the original PCT application PCT/SE00/01767.

Signed

Brita Nilsson

April 10, 2002

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: :
: Attention: PCT OFFICE
HOGLUND et al. :
: :
U.S. National Phase of PCT/SE00/01767 :
: :
Entry papers filed herewith March 15, 2002 :
: :
For: DNA CONSTRUCT AND ITS USE :

**PRELIMINARY AMENDMENT
AND INFORMATION DISCLOSURE STATEMENT**

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

The present application is the U.S. national phase of international application number PCT/SE00/01767. The following amendments pertain to the claims as amended.

Please note that the amended page 1 (new claim set) attached to the International Preliminary Examination Report (Annexes) and submitted herewith, have replaced the originally filed page 8 of the application. The claims to be examined and amended by this preliminary amendment are found on amended page 1 (of the new claim set).

Please amend the above-identified application as follows:

IN THE SPECIFICATION:

Please add the attached ABSTRACT OF THE DISCLOSURE to the application.

U.S. National Phase of PCT/SE00/01767

IN THE CLAIMS:

Please replace claims 3 and 5-6 with the following amended claims.

3(Amended). Transgenic oilseed plant cell according to claim 1, wherein the promoter is a napin promoter, the peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification is selected from the group consisting of peptides with, 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β -carotene hydroxylase, and acyl transferase activity.

5(Amended). Transgenic oilseed plant cell according to claim 1, wherein the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

6(Amended). Transgenic oilseed plant cell according to claim 1, wherein the cell expresses xanthophyll.

Please add the following new claims to the application.

10(New). Transgenic oilseed plant cell according to claim 2, wherein the promoter is a napin promoter, the peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification is selected from the group consisting of peptides with, 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β -carotene hydroxylase, and acyl transferase activity.

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11(New). Transgenic oilseed plant cell according to claim 2, wherein the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

12(New). Transgenic oilseed plant cell according to claim 3, wherein the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

13(New). Transgenic oilseed plant cell according to claim 4, wherein the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

14(New). Transgenic oilseed plant cell according to claim 10, wherein the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

15(New). Transgenic oilseed plant cell according to claim 2, wherein the cell expresses xanthophylls.

16(New). Transgenic oilseed plant cell according to claim 3, wherein the cell expresses xanthophylls.

17(New). Transgenic oilseed plant cell according to claim 4, wherein the cell expresses xanthophylls.

18(New). Transgenic oilseed plant cell according to claim 5, wherein the cell expresses xanthophylls.

19(New). Transgenic oilseed plant cell according to claim 10, wherein the cell expresses xanthophylls.

U.S. National Phase of PCT/SE00/01767

20(New). Transgenic oilseed plant cell according to claim 14, wherein the cell expresses xanthophylls.

U.S. National Phase of PCT/SE00/01767

REMARKS

Applicants have amended the claims in order to reduce the initial filing fee by deleting the multiple dependent claims from the application. Some of this subject matter has been reintroduced as dependent claims 10-20. Applicants retain the right to reintroduce any subject matter canceled by the present Amendment at any time during the prosecution of this application or any further application claiming benefit of this application.

Applicants have amended the application to substitute the originally filed page 8 with the amended claim set page 1 attached to the International Preliminary Examiner Report (Annexes) and included in the application as filed herewith. Also, an Abstract of the Disclosure has been added to the application.

Applicants are submitting herewith a copy of the International Search Report which issued on International Application No. PCT/SE00/01767, of which the present application is the U.S. national phase which was published in English. All of the publications cited in the International Search Report are listed on the attached Form PTO-1449. It is Applicants' understanding that, under the procedures of the PCT, copies of the cited publications will have been supplied to the U.S. Patent Office by the International Bureau. However, the Examiner is invited to contact the undersigned attorney if additional copies are necessary or would facilitate examination of the present application.

Otherwise, the Examiner is respectfully requested to return an initialed and dated copy of the attached Form PTO-1449 to confirm that all publications listed thereon have been considered and made officially of record in the file of this application.

Applicants understand that, under the procedures of the PCT, a copy of the priority document (SE 9903336-7, filed 17 September 1999) will have been supplied to

U.S. National Phase of PCT/SE00/01767

the U.S. Patent Office pursuant to Rule 17 of the PCT Regulations. It is therefore respectfully requested that the first Official Action in the present application contain an indication that the appropriate priority document is in the file of this application.

In view of the above amendments, an early action on the application is now in order and is most respectfully requested.

Respectfully submitted,
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REF:kdd
PA01.wpd

DATE: March 15, 2002

U.S. National Phase of PCT/SE00/01767

Marked-Up Version Showing Changes Made

IN THE CLAIMS:

Please replace claims 3 and 5-6 with the following amended claims.

3(Amended). Transgenic oilseed plant cell according to claim 1 [or 2], wherein the promoter is a napin promoter, the peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification is selected from the group consisting of peptides with, 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β -carotene hydroxylase, and acyl transferase activity.

5(Amended). Transgenic oilseed plant cell according to [any one of claims 1 - 5] claim 1, wherein the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

6(Amended). Transgenic oilseed plant cell according to [any one of claims 1 - 5] claim 1, wherein the cell expresses xanthophyll.

29295/BN

Abstract

A DNA construct comprising in the 5' to 3' direction of transcription operably linked a promoter region directing transcription to the seed of an oilseed plant, a
 5 nucleotide sequence coding for at least one peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification in an oilseed plant and a transcriptional termination region is disclosed. The DNA construct may additionally comprise a nucleotide sequence coding for a transit peptide directing the translated fusion polypeptide to the chloroplast of the oilseed plant. The peptide with enzyme
 10 activity is preferably a peptide with b-carotene C-4-oxygenase activity, e.g. from the alga *Haematococcus pluvialis*.

Comprised by the invention are also a transgenic oilseed plant cell, e.g. of rape, sunflower, soybean or mustard origin; transgenic oilseed plant-produced xanthophyll; transgenic oilseed plant-produced canthaxanthin; transgenic oilseed plant-produced
 15 astaxanthin; and transgenic oilseed plant-produced astaxanthin esters.

3/prls

1

DNA construct and its use.

The present invention relates to a new DNA construct for transformation into oilseed plants. The DNA construct comprises nucleotide sequences encoding peptides with enzyme activities necessary for the high-level production and esterification of keto group-containing xanthophylls in oilseed plants.

Background of the invention

Carotenoids are produced *de novo* by plants, fungi, algae and some bacteria. A number of biosynthetic steps are needed for the biological production of the carotenoids.

There are two chemically different groups of carotenoids, namely carotenes containing only carbon and hydrogen molecules and xanthophylls containing oxygen in the molecule in addition to carbon and hydrogen.

The xanthophylls, and particularly astaxanthin (3,3'-dihydroxy- β - β -carotene-4,4'-dione), are often colored pigments and are used as such or as anti-oxidants.

Carotenes are biological precursors for the production of the oxygen-containing xanthophylls. There are two types of enzymes responsible for the introduction of hydroxy groups and keto groups into the carotenes, namely hydroxylases and ketolases, respectively.

The keto group-containing xanthophyll astaxanthin, which has keto and hydroxy groups, is biosynthetically produced from beta-carotene.

Large-scale production of xanthophylls from natural sources is at present performed by AstaCarotene AB, Gustavsberg, Sweden, by cultivation of the alga *Haematococcus pluvialis* for the production of astaxanthin in esterified form.

It would be desirable to be able to produce keto group-containing xanthophylls particularly astaxanthin, in oilseed plants. Oilseed plants have naturally β -carotene hydroxylases but lack β -carotene C-4-oxygenase enzymes or ketolases.

Description of the invention

The present invention provides DNA constructs enabling and promoting production of keto group containing xanthophylls, especially astaxanthin, in oilseed plants, such as rape, sunflower, soybean and mustard. The DNA construct is transformed into the oilseed plant cell for expression of a protein or fused protein which has an enzyme activity enabling keto group insertion into a carotene or hydroxy carotene for the biosynthetic production of a keto group containing xanthophyll, such as cantaxanthin (β , β -carotene-4,4'-dione) and/or astaxanthin. Use is thus made of the biosynthetic pathway of the oilseed plant to

produce carotenoids. The naturally occurring synthesis of carotenoids involves a number of enzymes, namely 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β -carotene hydroxylase, and β -carotene C-4-oxygenase. Genes coding for peptides having these enzymatic activities may be inserted into the DNA construct of the invention, one or several per construct, to promote high-level production in the transgenic oilseed plant. In case only one enzyme coding gene is inserted per plant, two or more plants may be sexually interbred to produce plants containing all the desired enzyme activities.

Thus, the present invention is directed to a DNA construct comprising in the 5' to 3' direction of transcription operably linked a promoter region directing transcription to the seed of an oilseed plant, a nucleotide sequence coding for at least one peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification in an oilseed plant and a transcriptional termination region.

In a preferred embodiment of the invention the DNA construct additionally comprises between the promoter region and the nucleotide sequence coding for at least one peptide with enzyme activity a nucleotide sequence coding for a transit peptide directing the translated fusion polypeptide to the chloroplast of the oilseed plant.

The DNA construct is preferably such that the promoter is a napin promoter, the peptide with enzyme activity necessary for keto group containing xanthophyll production is selected from the group consisting of peptides with 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β -carotene hydroxylase, and β -carotene C-4-oxygenase activity. To promote esterification of astaxanthin a nucleotide sequence coding for a peptide with acyl transferase activity may be included in the group.

In a preferred embodiment of the DNA construct according to the invention the nucleotide sequence coding for a peptide with enzyme activity is a nucleotide sequence coding for a N-terminally truncated β -carotene C-4-oxygenase gene from the alga *Haematococcus pluvialis*.

An example of the DNA construct of the invention is presented in the sequence listing as SEQ ID NO:1 and in Fig.1.

The present invention is also directed to a transgenic oilseed plant cell comprising the DNA construct of the invention, and preferably the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

The invention is additionally directed to transgenic oilseed plant-produced xanthophyll, e.g. canthaxanthin and astaxanthin.

A preferred aspect of the invention is directed to transgenic oilseed plant-produced astaxanthin esters.

The present invention will now be illustrated with reference to the DNA construct disclosed in the sequence listing and in Fig.1, and the following description of embodiments. However, the invention is not limited to these exemplifications.

Short description of the drawings

Fig.1 illustrates the nucleotide sequence of the DNA construct comprising the napin promoter, the chloroplast localization signal, the N-terminally truncated β -carotene C-4-oxygenase gene and the termination sequence, and the deduced amino acid sequences of the transit peptide and the β -carotene C-4-oxygenase.

Description of embodiments

The invention is illustrated by production of astaxanthin in the seed of oilseed rape. The astaxanthin produced in the seed of the transgenic plant is extracted as part of the extracted oil. By use of conventionally used protocols for *Agrobacterium tumefaciens* mediated transformation such as described by (Hoekema et al.1983, An et al. 1986, Fry et al. 1987, DeBlock et al. 1988, Radke et al.1988, or Moloney et al. 1989) transgenic plants are produced having a chimeric DNA construct that is genetically inherited and is able to produce astaxanthin. The nucleotide sequence of the chimeric DNA construct consist of four parts of different genetic origin namely: (1) a promoter, (2) a localization signal, (3) a β -carotene C-4-oxygenase coding region and (4) a termination sequence.

The napin promoter directs transcription to the seed of oilseed rape (Stålberg et al 1996). This promoter was coupled to a localization signal similar but not identical to a transit peptide (TP) of Rbcs1a (Krebbers, 1988) that directs the translated product of a fused gene to the chloroplast. The promoter and the TP sequence were ligated to a part of the coding sequence of a ketolase gene BCK (Kajiwara et al. 1995). This enzyme oxygenates β -carotene to canthaxanthin, (Fraser et al. 1997). The chimeric DNA construct was then coupled to a suitable termination sequence, e.g. that of the *Agrobacterium tumefaciens* nopaline synthase gene (the nos 3' end)(Bevan et al. 1983), as illustrated in Fig.1.

Cellular storage of Astaxanthin

The storage of large amounts of free astaxanthin in plants will be difficult due to toxic effects of the molecule as it intercalates in the plant membranes. An effective esterification of astaxanthin to fatty acids enables storage of the esterified molecules in triacylglycerol containing oleosomes. Thus, an acyl transferase can be claimed to be of
 5 fundamental importance for the process, as is proteins that can mediate transport of different forms of astaxanthin from the chloroplast to the vesicles.

Sequences and oligonucleotides used in the construction of the DNA construct*1. Napin promoter (GeneBank ACCESSION No. J02798)*

10 This promoter sequence, a 1145 base pair fragment including the 5' leader sequence has a unique HindIII site at the 5' end. The 3' end was synthesized with an additionally 6 nucleotide BamHI site.

2. Transit peptide similar to RBCS1a (GeneBank ACCESSION No. X13611, X14565)

15 The transit peptide (TP) was amplified by PCR from -28 to the end of the transit cleavage aa=54/55 site of the Rbcs1a gene. The 5' end was synthesized with a BamHI site and similarly the 3' sequence was synthesized with a XbaI site. The two following oligonucleotides were used for the PCR amplification.

BamHI

20 5' primer: TP1 5'AGAC GGATCC TCAGTCACACAAAGAGTA 3'

SacI XbaI

3' primer: TP2 5'GTTC GAGCTC TCTAGA CATGCAGTTAACGC 3'

3. BCK (β -carotene C-4 oxygenase) (Genebank ACCESSION No. D45881)

25 The BCK fragment was amplified by PCR including a 5' XbaI site and was ligated to the TP already described. The 5' primer (BCK1) used for PCR, is homologous to the BCK sequence from nucleotide 264 and the 3' oligonucleotide (Ax40) ends with a stop codon and was synthesized with a SacI restriction site for cloning. The synthesized fragment was fused to the TP as shown in Fig 1.

30 Oligonucleotides used for PCR:

XbaI

5' primer: BCK1 5'ACAG TCTAGA ATGCCATCCGAGTCGTCA 3'

SacI

3' primer: AX40 5'CACCGAGCTCCATGACACTCTTGTGCAGA 3'

Description of SEQ ID NO:1 and SEQ ID NO:2

The sequences shown i Fig.1 are the same as the two sequences which are shown in the sequence listing.

The SEQ ID NO:1 is a nucleotide sequence composed of the following features:

5		Nucleotide No.
	Cloning site HindIII	1-6
	Napin Promoter	1-1145
	Cloning site BamHI	1146-1151
	Transit peptide leader	1152-1178
10	Transit peptide coding	1179-1347
	Cloning site XbaI	1348-1353
	β -carotene C-4-oxygenase	1354-2217
	β -carotene C-4-oxygenase 3' untranslated	2218-2266
	Cloning site SacI	2267-2272
15	Nopaline synthetase termination	2273-2536
	Cloning site EcoRI	2538-2543

The SEQ ID NO: 2 is a deduced amino acid sequence of the fusion protein of the transit peptide and the peptide with β -carotene C-4-oxygenase activity.

References

- An G, Watson BD, Chiang CC (1986), Transformation of tobacco, tomato, potato and
5 Arabidopsis-thaliana using a binary vector system. Plant Physiology 81 (1) 301-305.
- Bevan M, Barnes WM and Chilton MD (1983). Structure and transcription of the nopaline
synthase gene region of T-DNA. Nucleic Acids Res. 11 (2), 369-385 .
- 10 DeBlock M, DeBrouwer D, Tenning P (1989). Transformation of Brassica napus and Brassica
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transgenic plants Plant Physiology 91:2, 694-701.
- Fraser PD, Miura Y, Misawa N, (1997). In vitro characterization of astaxanthin biosynthetic
15 enzymes. J Biol Chem. Mar 7;272(10):6128-35.
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25 1.7 S storage protein, napin, from Brassica napus. J. Biol. Chem. 262 (25), 12196-12201.
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from Haematococcus pluvialis, and astaxanthin synthesis in Escherichia coli Plant Mol. Biol.
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- Radke SE, Andrews BM, Moloney MM, Crouch ML, Kridl JC, Knauf VC (1988),
Transformation of *Brassica napus* using *Agrobacterium tumefaciens* – Developmentally
10 regulated Expression of a reintroduced napin gene. TAG, 75: (5) 685-694 .

Pua E-C, Mehra-Palta A, Nagy F and Chua N-H, (1987). Transgenic plants of *Brassica napus*.
Biotechnology vol 5, 815-817.

- 15 Stålberg K, Ellerstöm M, Ezcurra I, Ablov S, Rask L (1996). Disruption of an overlapping E-box/ABRE motif abolished high transcription of the napA storage-protein promoter in transgenic *Brassica napus* seeds. Planta 199(4):515-9.

Claims

1. Transgenic oilseed plant cell containing a DNA construct comprising in the 5' to 3' direction of transcription operably linked a promoter region directing transcription to the seed of the oilseed plant, a nucleotide sequence coding for a transit peptide directing the translated fusion polypeptide to the chloroplast of the oilseed plant, a 5'-truncated beta-carotene C-4-oxygenase gene from the alga *Haematococcus pluvialis* and a transcriptional termination region.

2. Transgenic oilseed plant cell according to claim 1, wherein the cell additionally contains at least one DNA construct selected from DNA constructs comprising in the 5' to 3' direction of transcription operably linked a promoter region directing transcription to the seed of the oilseed plant, a nucleotide sequence coding for a transit peptide directing the translated fusion polypeptide to the chloroplast of the oilseed plant, a nucleotide sequence coding for at least one peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification in the oilseed plant and a transcriptional termination region.

3. Transgenic oilseed plant cell according to claim 1 or 2, wherein the promoter is a napin promoter, the peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification is selected from the group consisting of peptides with, 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β -carotene hydroxylase, and acyl transferase activity.

4. Transgenic oilseed plant cell according to claim 1, wherein the nucleotide sequence of the DNA construct is SEQ ID NO:1.

5. Transgenic oilseed plant cell according to any one of claims 1 - 5, wherein the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

6. Transgenic oilseed plant cell according to any one of claims 1 - 5, wherein the cell expresses xanthophylls.

7. Transgenic oilseed plant cell according to claim 6, wherein a xanthophyll is canthaxanthin.

8. Transgenic oilseed plant cell according to claim 6, wherein a xanthophyll is astaxanthin.

9. Transgenic oilseed plant cell according to claim 8, wherein the astaxanthin comprises astaxanthin esters.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
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(54) Title: DNA CONSTRUCT AND ITS USE

(57) Abstract: A DNA construct comprising in the 5' to 3' direction of transcription operably linked a promoter region directing transcription to the seed of an oilseed plant, a nucleotide sequence coding for at least one peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification in an oilseed plant and a transcriptional termination region is disclosed. The DNA construct may additionally comprise a nucleotide sequence coding for a transit peptide directing the translated fusion polypeptide to the chloroplast of the oilseed plant. The peptide with enzyme activity is preferably a peptide with β -carotene C-4-oxygenase activity, e.g. from the alga *Haematococcus pluvialis*. Comprised by the invention are also a transgenic oilseed plant cell, e.g. of rape, sunflower, soybean or mustard origin, and a transgenic oilseed plant-produced xanthophyll, such as canthaxanthin or astaxanthin, and also astaxanthin esters.

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Napin promoter

AAGCTTTCTTCATCGGTGATTGATTCCTTTAAAGACTTATGTTTCTTATCTTGCTTCTGA
GGCAAGTATTCAGTTACCAGTTACCACTTATATTCTGGACTTTCTGACTGCATCCTCATT
TTTCCAACATTTTAAATTTCACTATTGGCTGAATGCTTCTTCTTTGAGGAAGAAACAATT
CAGATGGCAGAAATGTATCAACCAATGCATATATACAAATGTACCTCTTGTTCTCAAAAC
ATCTATCGGATGGTTCCATTTGCTTTGTCATCCAATTAGTGACTACTTTATATTATTAC
TCCTCTTTATTACTATTTTCATGCGAGGTTGCCATGTACATTATATTGTAAGGATTGAC
GCTATTGAGCGTTTTTCTTCAATTTTCTTTATTTTAGACATGGGTATGAAATGTGTGTTA
GAGTTGGGTTGAATGAGATATACGTTCAAGTGAAGTGGCATAACCGTTCTCGAGTAAGGAT
GACCTACCCATTCTTGAGACAAATGTTACATTTTAGTATCAGAGTAAAATGTGTACCTAT
AACTCAAATTCGATTGACATGTATCCATTCAACATAAAATTAAACCAGCCTGCACCTGCA
TCCACATTTCAAGTATTTTCAAACCGTTCGGCTCCTATCCACCGGGTGTAACAAGACGGA
TTCCGAATTTGGAAGATTTTGACTCAAATTCCCAATTTATATTGACCGTGACTAAATCAA
CTTTAACTTCTATAATTCTGATTAAGCTCCCAATTTATATTCCCAACGGCACTACCTCCA
AAATTTATAGACTCTCATCCCCTTTTAAACCAACTTAGTAAACGTTTTTTTTTTTAAATTT
TATGAAGTTAAGTTTTTACCTTGTTTTTAAAAGAATCGTTCATAAGATGCCATGCCAGA
ACATTAGCTACACGTTACACATAGCATGCAGCCGCGGAGAATTGTTTTTCTTCGCCACTT
GTCACTCCCTTCAAACACCTAAGAGCTTCTCTCTCACAGCACACACATACAATCACATGC
GTGCATGCATTATTACACGTGATCGCCATGCAAATCTCCTTTATAGCCTATAAATTA
ACTCATCCGCTTCACTCTTTACTCAAACCAAACCTCATCAATACAAACAAGATTAAAAACATA

End -28 untranslated leader TP start
CACGAGGATCCTCAGTCACACAAAGAGTAAAGAAGAACAATGGCTTCCTCTATGCTCTCT
M A S S M L S

TCCGCTACTATGGTTGCCTCTCCGGCTCAGGCCACTATGGTCGCTCCTTTCAACGGACTT
S A T M V A S P A Q A T M V A P F N G L

AAGTCCTCCGCTGCCTTCCCAGCCACCCGCAAGGCTAACAACGACATTACTTCCATCACA
K S S A A F P A T R K A N N D I T S I T

FIG.1

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TP End

C-4-Oxygenase

AGCAACGGCGGACGCGTTAACTGCATGTCTAGAATGCCATCCGAGTCGTCAGACGCAGCT
S N G G R V N C M S R M P S E S S D A A

CGTCCTGCGCTAAAGCACGCCTACAAACCTCCAGCATCTGACGCCAAGGGCATCACGATG
R P A L K H A Y K P P A S D A K G I T M

GCGCTGACCATCATTGGCACCTGGACCGCAGTGTTTTTACACGCAATATTTCAAATCAGG
A L T I I G T W T A V F L H A I F Q I R

CTACCGACATCCATGGACCAGCTTCACTGGTTGCCTGTGTCCGAAGCCACAGCCCAGCTT
L P T S M D Q L H W L P V S E A T A Q L

TTGGGCGGAAGCAGCAGCCTACTGCACATCGCTGCAGTCTTCATTGTACTTGAGTTCCTG
L G G S S S L L H I A A V F I V L E F L

TACACTGGTCTATTCATCACACACATGACGCAATGCATGGCACCATAGCTTTGAGGCAC
Y T G L F I T T H D A M H G T I A L R H

AGGCAGCTCAATGATCTCCTTGGCAACATCTGCATATCACTGTACGCCTGGTTTGACTAC
R Q L N D L L G N I C I S L Y A W F D Y

AGCATGCTGCATCGCAAGCACTGGGAGCACCACAACCATACTGGCGAAGTGGGGAAAGAC
S M L H R K H W E H H N H T G E V G K D

CCTGACTTCCACAAGGGAAATCCCGGCCTTGTCCCCTGGTTCGCCAGCTTCATGTCCAGC
P D F H K G N P G L V P W F A S F M S S

TACATGTCCCTGTGGCAGTTTGCCCGGCTGGCATGGTGGGCAGTGGTGATGCAAATGCTG
Y M S L W Q F A R L A W W A V V M Q M L

GGGGCGCCCATGGCAAATCTCCTAGTCTTCATGGCTGCAGCCCCAATCTTGTGAGCATTC
G A P M A N L L V F M A A A P I L S A F

CGCCTCTTCTACTTCGGCACTTACCTGCCACACAAGCCTGAGCCAGGCCCTGCAGCAGGC
R L F Y F G T Y L P H K P E P G P A A G

TCTCAGGTGATGGCCTGGTTCAGGGCCAAGACAAGTGAGGCATCTGATGTGATGAGTTTC
S Q V M A W F R A K T S E A S D V M S F

CTGACATGCTACCACTTTGACCTGCACTGGGAGCACCACAGATGGCCCTTTGCCCCCTGG
L T C Y H F D L H W E H H R W P F A P W

TGGCAGCTGCCCCACTGCCGCCGCCTGTCCGGGCGTGGCCTGGTGCCTGCCTTGGCATGA
W Q L P H C R R L S G R G L V P A L A *

C-4 oxygenase Stop

FIG.1 (cont.)

10/07/04 12 10/07/04 12

10/0/04 12

3/3

C-4 oxygenase untranslated region Nos term
CCTGGTCCCTCCGCTGGTGACCCAGCGTCTGCACAAGAGTGTTCATGGAGCTCGAATTTCC
CCGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTG
CGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAAT
GCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAAT
ACGCGATAGAAAACAAAATATAGCGCGCAAAGTAGGATAAATTATCGCGCGCGGTGTCAT
end
CTATGTTACTAGATCGGGAATTC

Fig.1 (cont.)

ATTORNEY/DOCKET NO: HOGL3001/REF

DECLARATION FOR PATENT APPLICATION AND APPOINTMENT OF ATTORNEY

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name; I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention (Design, if applicable) entitled:

DNA CONSTRUCT AND ITS USE

the specification of which (check one):

☐ is attached hereto, or ☒ was filed on: **13 September 2000** as PCT International Application Number: **PCT/SE00/01767**

and (if applicable) was amended on:

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56. I hereby claim foreign priority benefits under Title 35, United States Code §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

PRIOR FOREIGN APPLICATION(S)			PRIORITY CLAIMED	
Number	Country	Day/Month/Year Filed	Yes	No
9903336-7	Sweden	17 September 1999	X	

☐ Additional Priority Application(s) Listed on Following Page(s)

I HEREBY CLAIM THE BENEFIT UNDER TITLE 35 U.S. CODE §119(E) OF ANY U.S. PROVISIONAL APPLICATIONS LISTED BELOW.	
Application Number	Day/Month/Year Filed

☐ Additional Provisional Application(s) Listed on Following Page(s)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating The United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application:

Application Number	Filing Date	Status - Patented, Pending or Abandoned

☐ Additional US/PCT Priority Application(s) listed on Following Page(s)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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DATE 020304	SIGNATURE <i>Anna-Stina Hoglund</i>	

☒ See following page(s) for additional joint inventors.

(04AUG1998)

ATTORNEY/DOCKET NO: HOGL3001/REF

CONTINUATION OF DECLARATION FOR PATENT APPLICATION AND APPOINTMENT OF ATTORNEY

Page 2

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DATE	SIGNATURE

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DATE	SIGNATURE

FULL NAME OF JOINT INVENTOR	CITIZENSHIP
RESIDENCE ADDRESS	POST OFFICE ADDRESS IS THE SAME AS RESIDENCE ADDRESS UNLESS OTHERWISE SHOWN BELOW
DATE	SIGNATURE

☐ See following pages for additional joint inventors/priority applications.

SEQUENCE LISTING

<110> AstaCarotene AB

<120> DNA construct and its use

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<223> Description of Artificial Sequence: napin promoter
+ chloroplast localization signal + beta-carotene C-4 oxygenase
coding sequence + termination sequence

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Thr Met Ala Leu Thr Ile Ile Gly Thr Trp Thr Ala Val Phe Leu His
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 Ser Leu Trp Gln Phe Ala Arg Leu Ala Trp Trp Ala Val Val Met Gln
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atg ctg ggg gcg ccc atg gca aat ctc cta gtc ttc atg gct gca gcc 1962
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 250 255 260

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 Pro Ile Leu Ser Ala Phe Arg Leu Phe Tyr Phe Gly Thr Tyr Leu Pro
 265 270 275

cac aag cct gag cca ggc cct gca gca ggc tct cag gtg atg gcc tgg 2058
 His Lys Pro Glu Pro Gly Pro Ala Ala Gly Ser Gln Val Met Ala Trp
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 Pro Trp Trp Gln Leu Pro His Cys Arg Arg Leu Ser Gly Arg Gly Leu
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 <223> Description of Artificial Sequence: deduced fusion protein of
 transit peptide + peptide with beta-carotene C-4 oxygenase activity

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Gln Ala Thr Met Val Ala Pro Phe Asn Gly Leu Lys Ser Ser Ala Ala
 20 25 30

Phe Pro Ala Thr Arg Lys Ala Asn Asn Asp Ile Thr Ser Ile Thr Ser
 35 40 45

Asn Gly Gly Arg Val Asn Cys Met Ser Arg Met Pro Ser Glu Ser Ser
 50 55 60

Asp Ala Ala Arg Pro Ala Leu Lys His Ala Tyr Lys Pro Pro Ala Ser
 65 70 75 80

Asp Ala Lys Gly Ile Thr Met Ala Leu Thr Ile Ile Gly Thr Trp Thr
 85 90 95

Ala Val Phe Leu His Ala Ile Phe Gln Ile Arg Leu Pro Thr Ser Met
 100 105 110

Asp Gln Leu His Trp Leu Pro Val Ser Glu Ala Thr Ala Gln Leu Leu
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Gly Gly Ser Ser Ser Leu Leu His Ile Ala Ala Val Phe Ile Val Leu
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Glu Phe Leu Tyr Thr Gly Leu Phe Ile Thr Thr His Asp Ala Met His
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Gly Thr Ile Ala Leu Arg His Arg Gln Leu Asn Asp Leu Leu Gly Asn
 165 170 175

Ile Cys Ile Ser Leu Tyr Ala Trp Phe Asp Tyr Ser Met Leu His Arg
 180 185 190

Lys His Trp Glu His His Asn His Thr Gly Glu Val Gly Lys Asp Pro
 195 200 205

Asp Phe His Lys Gly Asn Pro Gly Leu Val Pro Trp Phe Ala Ser Phe
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Met Ser Ser Tyr Met Ser Leu Trp Gln Phe Ala Arg Leu Ala Trp Trp
 225 230 235 240

Ala Val Val Met Gln Met Leu Gly Ala Pro Met Ala Asn Leu Leu Val
 245 250 255

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Gly Thr Tyr Leu Pro His Lys Pro Glu Pro Gly Pro Ala Ala Gly Ser
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Gln Val Met Ala Trp Phe Arg Ala Lys Thr Ser Glu Ala Ser Asp Val
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Met Ser Phe Leu Thr Cys Tyr His Phe Asp Leu His Trp Glu His His
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cacgaggatc	ctcagtcaca	caaagagtaa	agaagaaca	atg gct tcc tct atg Met Ala Ser Ser Met 1 5		1194
ctc tct tcc gct act atg gtt gcc tct ccg gct cag gcc act atg gtc Leu Ser Ser Ala Thr Met Val Ala Ser Pro Ala Gln Ala Thr Met Val	10	15	20			1242
gct cct ttc aac gga ctt aag tcc tcc gct gcc ttc cca gcc acc cgc Ala Pro Phe Asn Gly Leu Lys Ser Ser Ala Ala Phe Pro Ala Thr Arg	25	30	35			1290
aag gct aac aac gac att act tcc atc aca agc aac ggc gga cgc gtt Lys Ala Asn Asn Asp Ile Thr Ser Ile Thr Ser Asn Gly Gly Arg Val	40	45	50			1338
aac tgc atg tct aga atg cca tcc gag tcg tca gac gca gct cgt cct Asn Cys Met Ser Arg Met Pro Ser Glu Ser Ser Asp Ala Ala Arg Pro	55	60	65			1386
gcg cta aag cac gcc tac aaa cct cca gca tct gac gcc aag ggc atc Ala Leu Lys His Ala Tyr Lys Pro Pro Ala Ser Asp Ala Lys Gly Ile	70	75	80			1434
acg atg gcg ctg acc atc att ggc acc tgg acc gca gtg ttt tta cac Thr Met Ala Leu Thr Ile Ile Gly Thr Trp Thr Ala Val Phe Leu His	90	95	100			1482
gca ata ttt caa atc agg cta ccg aca tcc atg gac cag ctt cac tgg Ala Ile Phe Gln Ile Arg Leu Pro Thr Ser Met Asp Gln Leu His Trp	105	110	115			1530
ttg cct gtg tcc gaa gcc aca gcc cag ctt ttg ggc gga agc agc agc Leu Pro Val Ser Glu Ala Thr Ala Gln Leu Leu Gly Gly Ser Ser Ser	120	125	130			1578
cta ctg cac atc gct gca gtc ttc att gta ctt gag ttc ctg tac act Leu Leu His Ile Ala Ala Val Phe Ile Val Leu Glu Phe Leu Tyr Thr	135	140	145			1626

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 Gly Leu Phe Ile Thr Thr His Asp Ala Met His Gly Thr Ile Ala Leu
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 Arg His Arg Gln Leu Asn Asp Leu Leu Gly Asn Ile Cys Ile Ser Leu
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 tac gcc tgg ttt gac tac agc atg ctg cat cgc aag cac tgg gag cac 1770
 Tyr Ala Trp Phe Asp Tyr Ser Met Leu His Arg Lys His Trp Glu His
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 His Asn His Thr Gly Glu Val Gly Lys Asp Pro Asp Phe His Lys Gly
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 Asn Pro Gly Leu Val Pro Trp Phe Ala Ser Phe Met Ser Ser Tyr Met
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 Ser Leu Trp Gln Phe Ala Arg Leu Ala Trp Trp Ala Val Val Met Gln
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 His Lys Pro Glu Pro Gly Pro Ala Ala Gly Ser Gln Val Met Ala Trp
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 Phe Arg Ala Lys Thr Ser Glu Ala Ser Asp Val Met Ser Phe Leu Thr
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 Cys Tyr His Phe Asp Leu His Trp Glu His His Arg Trp Pro Phe Ala
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Phe	Met	Ala	Ala 260	Ala	Pro	Ile	Leu	Ser 265	Ala	Phe	Arg	Leu	Phe 270	Tyr	Phe
Gly	Thr	Tyr 275	Leu	Pro	His	Lys	Pro 280	Glu	Pro	Gly	Pro	Ala 285	Ala	Gly	Ser
Gln	Val 290	Met	Ala	Trp	Phe	Arg 295	Ala	Lys	Thr	Ser	Glu 300	Ala	Ser	Asp	Val
Met 305	Ser	Phe	Leu	Thr	Cys 310	Tyr	His	Phe	Asp	Leu 315	His	Trp	Glu	His	His 320
Arg	Trp	Pro	Phe	Ala 325	Pro	Trp	Trp	Gln	Leu 330	Pro	His	Cys	Arg	Arg 335	Leu
Ser	Gly	Arg	Gly 340	Leu	Val	Pro	Ala	Leu 345	Ala						